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Guanylin: An endogenous activator of intestinal guanylate cyclase

(intestine/cyclic GMP/heat-stable enterotoxin/diarrhea/peptide)

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ABSTRACT **Intestinal guanylate cyclase mediates the action of the heat-stable enterotoxin to cause a decrease in intestinal fluid absorption and to increase chloride secretion, ultimately causing diarrhea. An endogenous ligand that acts on this guanylate cyclase has not previously been found. To search for a potential endogenous ligand, we utilized T84 cells, a human colon carcinoma-derived cell line, in culture as a bioassay. This cell line selectively responds to the toxin in a very sensitive manner with an increase in intracellular cyclic GMP. In the present study, we describe the purification and structure of a peptide from rat jejunum that activates this enzyme. This peptide, which we have termed guanylin, is composed of 15 amino acids and has the following amino acid sequence, PNTCEICAYAACTGC, as determined by automated Edman degradation sequence analysis and electrospray mass spectrometry. Analysis of the amino acid sequence of this peptide reveals a high degree of homology with heat-stable enterotoxins. Solid-phase synthesis of this peptide confirmed that it stimulates increases in T84 cyclic GMP levels. Guanylin required oxidation for expression of bioactivity and subsequent reduction of the oxidized peptide eliminated the effect on cyclic GMP, indicating a requirement for cysteine disulfide bond formation. Synthetic guanylin also displaces heat-stable enterotoxin binding to cultured T84 cells. Based on these data, we propose that guanylin is an activator of intestinal guanylate cyclase and that it stimulates this enzyme through the same receptor binding region as the heat-stable enterotoxins.**

Guanylate cyclase is comprised of a group of proteins that share a common enzymatic function of producing cyclic GMP but differ quite remarkably in their selectivity toward activation by ligands. The three major types of guanylate cyclase are the soluble, particulate, and intestinal [cytoskeletal-associated particulate or heat-stable enterotoxin (STa)-sensitive] forms and each is regulated by different ligands (1, 2). Activation of the soluble guanylate cyclase occurs in response to nitric oxide, whereas activation of the particulate enzyme occurs in response to the natriuretic peptides (atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide) (1, 2). An endogenous activator of the intestinal guanylate cyclase has not previously been identified. However, the STa from *Escherichia coli* is known to selectively activate this form of the enzyme (3, 4). The intestinal form is predominantly found in the intestinal epithelial cells with the largest number of receptors oriented toward the lumen (1, 2). Recently, it has been cloned and expressed from rat small intestinal mucosa (5). This enzyme is characterized by an extracellular receptor binding region, a transmembrane region, an intracellular protein kinase-like region, and a cyclase catalytic domain (5).

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Pathogenic strains of *E. coli* and other bacteria produce a family of heat-stable enterotoxins (STs) that activate intestinal guanylate cyclase. STs are acidic peptides that contain 18 or 19 amino acids with six cysteines and three disulfide bridges that are required for full expression of bioactivity (6). The increase of intestinal epithelial cyclic GMP elicited by STs is thought to cause a decrease in water and sodium absorption and an increase in chloride secretion (7, 8). These changes in intestinal fluid and electrolyte transport then act to cause secretory diarrhea. In developing countries, the diarrhea resulting from STs causes many deaths, particularly in the infant population (9). STs are also considered a major cause of traveler's diarrhea in developed countries (10). They have also been reported to be a leading cause of morbidity and death in domestic animals (11).

In the present study, we designed a bioassay to search for a potential endogenous ligand that activates the intestinal guanylate cyclase. This bioassay is based on the demonstration that T84 cells in culture respond to ST in a selective and sensitive manner with graded increases of intracellular cyclic GMP. This bioassay revealed that the intestine as well as the kidney possessed an active material. Purification of this material from the rat intestine was accomplished and the structure was determined to be a 15-amino acid peptide with 4 cysteines that must be disulfide-linked for bioactivity. The peptide, termed guanylin, also possesses a high degree of homology with STs.

MATERIALS AND METHODS

Cell Culture. A cultured human colon carcinoma cell line (T84) was obtained from the American Type Culture Collection at passage 52. Cells were grown to confluency in 24-well culture plates with a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 international units of penicillin per ml, and 100 μg of streptomycin per ml. Cells were used at passages 54–60.

Cyclic GMP Determination. Monolayers of T84 cells in 24-well plates were washed twice with 1 ml of DMEM per ml and then incubated at 37°C for 10 min with 0.5 ml of DMEM containing 1 mM isobutylmethylxanthine (IBMX), a cyclic nucleotide phosphodiesterase inhibitor. Agents and fractions were then added for the indicated time as described in *Results*. The media was then aspirated and the reaction was terminated by the addition of ice-cold 0.5 ml of 0.1 M HCl. Aliquots were then evaporated to dryness under nitrogen and resuspended in 5 mM sodium acetate buffer (pH 6.4). The

Abbreviations: IBMX, isobutylmethylxanthine; TFA, trifluoroacetic acid; C_{18}, octadecasilyl; STa, heat-stable enterotoxin; ST, heat-stable enterotoxin; DTT, dithiothreitol; PTH, phenylthiohydantoin.

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samples were subsequently measured for cyclic GMP by RIA as described by Steiner et al. (12).

Purification of Guanylin. Rat jejunums flushed of luminal contents with 50 ml of saline and immediately placed on dry ice were obtained from Bioproducts for Science (Indianapolis). The jejunums were thawed, minced, and boiled for 10 min in 1 M acetic acid. The extract was centrifuged at 20,000 × g for 20 min at 4°C. The resulting supernatant was filtered and applied to an octadecasilyl (C_{18}) Sep-Pak (Waters). The column was washed with 10% acetonitrile/0.1% trifluoroacetic acid (TFA)/H_2O and eluted with 60% acetonitrile/0.1% TFA/H_2O. The eluted peptide fraction was lyophilized and resuspended in 50 ml of distilled H_2O containing 0.8% ampholytes, pH range 3–10, and applied to a preparative isoelectric focusing cell (Rotofor, Bio-Rad). The sample was focused for 150 min at 12 W constant power. The fractions were harvested, pH determined, and bioassayed. The active fractions, which focused around pH 3.8, were then refocused under similar conditions and the resulting active fractions were lyophilized. The sample was then resuspended in 1 ml of 10% acetonitrile/0.1% TFA/H_2O, applied to a C_{18} semipreparative HPLC column (Vydac, Hesperia, CA), and eluted at a flow rate of 3 ml/min. The following gradient was used to fractionate the sample: 10% acetonitrile, 0.1% TFA to 30% acetonitrile, 0.1% TFA in 180 min. The active fraction was then determined by bioassay and lyophilized. This sample was resuspended in 1 ml of 10% acetonitrile/0.1% TFA/H_2O and applied to a phenyl analytical HPLC column (Vydac, Hesperia, CA). The conditions for elution were similar to that described above for the semipreparative column except the flow was 1 ml/min. The active fraction was lyophilized and then resuspended in 1 ml of 10% acetonitrile/0.1% TFA/H_2O. The sample was then applied to a C_{18} analytical HPLC column (Vydac) and eluted according to the above description for the phenyl column. The active fraction was identified by bioassay and lyophilized. The sample was reconstituted in 1 ml of 10% acetonitrile/0.1% TFA/H_2O, reapplied to the analytical C_{18} column, and eluted by a gradient of 10% acetonitrile/10 mM ammonium acetate/H_2O, pH 6.2, to 30% acetonitrile/10 mM ammonium acetate/H_2O, pH 6.2, in 180 min. The active fraction was lyophilized and reconstituted in 0.05 ml of 0.1% TFA/H_2O. The sample was then applied to a C_8 microbore column and eluted by an increasing gradient of 0.33%/min of acetonitrile/0.1% TFA/H_2O. Two separate batches of purified peptide were then subjected to sequence analysis.

N-Terminal Protein Sequence Analysis. Automated Edman degradation chemistry was used to determine the NH_2-terminal protein sequence. An Applied Biosystems model 470A gas-phase sequencer was employed for the degradations (13) using the standard sequencer cycle 03RPTH. The respective phenylthiohydantoin (PTH) amino acid derivatives were identified by reverse-phase HPLC analysis in an on-line fashion employing an Applied Biosystems model 120A PTH analyzer fitted with a Brownlee 2.1-mm i.d. PTH C_{18} column. On-sequencer pyridylethylation was performed as outlined by Kruft et al. (14). The PTH derivative of pyridylethlycysteine was identified by HPLC as eluting slightly prior to the PTH derivative of methionine.

Electrospray Mass Spectrometry. Individual samples of native and synthetic guanylin were purified by microbore C_8 reverse-phase HPLC (Brownlee Aquapore RP-300 7-μm column, P. J. Cobert, St. Louis, MO) and eluting fractions of the peptides were collected and concentrated to ≈8 pmol/μl for mass analysis. Sample solutions were introduced to the mass spectrometer via injection into a stream of acetonitrile/H_2O/TFA, 1000:1000:1, vol/vol/vol, which continuously flowed to the mass spectrometer at a flow of 10 μl/min. Three microliters of each of the concentrated guanylin samples was injected to obtain the results that are presented in this paper. A Sciex API III triple-quadrupole mass spectrometer (Thornhill, Ontario, Canada) equipped with an atmospheric pressure ion source was used to sample positive ions produced from an electrospray interface (15). Mass analysis of sample ions was accomplished by scanning the first quadrupole in 1 atomic mass unit increments from 1000 to 2400 atomic mass units in ≈3 s and passing mass-selected ions through the second and third quadrupoles operated in the rf-only mode to the multiplier. For maximum sensitivity, the mass resolution of the quadrupole mass analyzer was set so that ion signals were ≈2 atomic mass units wide at half peak height, but the centroid of the ion signal still represented the correct mass of the ion. Comparison of the oxidized and reduced guanylin molecular ion region was made by scanning the quadrupole mass analyzer in 0.1 atomic mass unit steps from 1510 to 1525 atomic mass units in 2 s. Mass spectra of the guanylin samples were averaged over all of the scans that were acquired during elution of the 3-μl sample solution.

Binding Assay. ^{125}I-labeled STa (^{125}I-STa) was prepared by the Iodo-Gen method (16). T84 cell monolayers were washed twice with 1 ml of DMEM and then incubated for 30 min at 37°C in 0.5 ml of DMEM with ^{125}I-STa (amino acids 5–18) (100,000 cpm per well) and either guanylin or 100 nM STa. The cells were then washed four times with 1 ml of DMEM and solubilized with 0.5 ml of 1 M NaOH per well. This volume was transferred to tubes and assayed for radioactivity by a γ counter. Results are expressed as the percentage specifically bound.

Chemical Synthesis of Guanylin. Guanylin was synthesized by the solid-phase method (17) with an Applied Biosystems 430A peptide synthesizer on Cys(4-CH_3Bzl)-OCH_2-phenylacetamidomethyl resin using double coupling cycles to ensure complete coupling at each step. Coupling was effected with preformed symmetrical anhydride of *tert*-butoxycarbonyl amino acids (Applied Biosystems), and peptides were cleaved from the solid support in hydrogen fluoride/dimethylsulfide/anisole/*p*-thiocresol, 8:1:1:0.5, vol/vol/wt, at 0°C for 60 min. Peptides were cyclized using dimethylsulfoxide as described by Tam et al. (18). Peptides were purified by successive reverse-phase chromatography on a 45 × 300 mm Vydac C_{18} column and on a 19 × 150 mm μBondapak C_{18} column, using a gradient of 10–30% acetonitrile in 0.5% TFA. The structures and purity of the synthetic peptides were verified by fast atom bombardment mass spectrometry, amino acid analysis, and gas-phase sequence analysis.

RESULTS

Initial characterization of the T84 cell response indicated that these cells were very sensitive to STa with a concentration of 10^{-10} M eliciting a 4-fold increase in cyclic GMP. The cells also displayed a remarkable range, with a maximal response of STa (10^{-7} M) eliciting a >1000-fold increase in cyclic GMP. Furthermore, we failed to detect an effect of either sodium nitroprusside (10^{-3} M) or atrial natriuretic peptide (10^{-6} M) on cyclic GMP levels, suggesting that the T84 serves as a selective bioassay for agents that activate the intestinal guanylate cyclase. Various rat tissues were surveyed as sources for T84 cell cyclic GMP agonist activity, and jejunum and kidney were found to possess activity while liver, brain, pancreas, spleen, lung, and testes lacked detectable activity (Fig. 1). We also observed that rat embryonic intestine possessed a similar activity. Treatment of the T84 cells with 10% of embryonic intestinal extract increased the cyclic GMP from a basal level of 120 ± 10 fmol per well to 270 ± 10 fmol per well (mean ± SE).

Purification of the adult rat jejunal bioactivity was accomplished by the processing scheme described in *Materials and Methods*. Briefly, following acid boiling and extraction by a

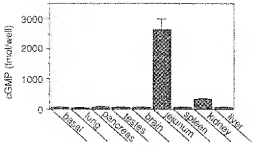

FIG. 1. Effect of extracts from various tissues on T84 cyclic GMP levels. Acid extracts were prepared from 1 g of tissue and 10% of each extract was applied to IBMX-treated cells. Values are means ± SE ($n = 3$).

C_{18} reverse-phase matrix, the material was fractionated on a preparative isoelectric focusing cell, which resulted in a 200-fold purification and indicated that the isoelectric point was about 3.8. Refocusing of the active fraction resulted in a further 5- to 10-fold purification. The active fraction was then purified to homogeneity by a series of reverse-phase HPLC steps, including a semipreparative C_{18} column, a phenyl column, two separations on a C_{18} column utilizing different ion-pairing reagents, and final purification on a microbore C_8 column (Fig. 2).

Preliminary experiments suggested that the material was a low molecular weight peptide; therefore the material was subjected to N-terminal protein sequence analysis and to electrospray mass spectrometric analysis. The combination of the data derived from these two techniques yielded the complete sequence for guanylin: Pro-Asn-Thr-Cys-Glu-Ile-Cys-Ala-Tyr-Ala-Ala-Cys-Thr-Gly-Cys. The N-terminal sequence through 14 places was determined by two independent gas-phase sequencing experiments. The C-terminal amino acid was deduced from data obtained by electrospray mass spectroscopy. The initial results yielded a sequence in which no PTH amino acid derivative was observed at positions 4, 7, and 12. Since cysteine residues cannot be positively identified during gas-phase sequencing without reduction and alkylation, the lack of a PTH amino acid derivative at these positions suggested the presence of cysteine residues. For complete verification, the putative cysteine residues of guanylin were pyridylethylated and the peptide was resequenced. The subsequent N-terminal gas-phase sequence analysis verified cysteine residues at positions 4, 7, and 12. Further primary structure information was obtained by electrospray mass spectrometry. The electrospray mass spectrum of native guanylin (Fig. 3*A*) contains an ion signal at m/z 1516 that corresponds to the protonated peptide. This assignment is 103 atomic mass units higher than the mass expected for the peptide whose sequence was obtained by gas-phase sequence analysis. This difference of 103 atomic mass units is consistent with an additional disulfide-linked cysteine or with a threonine at the C terminus of the peptide. Reduction of the disulfide bonds of guanylin with dithiothreitol (DTT) resulted in a 4 atomic mass unit increase in molecular weight of the peptide (Fig. 3 *B* and *C*), indicating that it contains two disulfide bonds. Therefore, since there are only three cysteines in the original 14 N-terminal amino acids, the 103 atomic mass unit difference must result from an additional C-terminal cysteine that is disulfide-linked to one of the other three cysteines in the guanylin sequence.

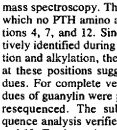

FIG. 2. Final purification of guanylin by C_8 reverse-phase microbore HPLC. Chromatographic peaks (A_{214}, 0.1 absorbance unit full scale) were collected and measured for activity. The active peak (55% of a full-scale response) is indicated by shading, with 1% of the fraction giving a 10-fold increase in cyclic GMP.

The resulting full amino acid sequence of the peptide was compared with all other proteins in the GenBank, National Biomedical Research Foundation, and SwissProt databases by a computer-based search. This search revealed that guanylin has homology with the STs, with the greatest homology identified in the cysteine-rich regions of the molecules (19, 20). The distinctive difference between guanylin and the STs is that guanylin possesses four cysteines with two disulfide-linked bridges while all of the known STs have six cysteines with three disulfide-linked bridges (Fig. 4).

Chemical synthesis of guanylin based on the experimentally derived sequence resulted in three different HPLC fractions following oxidation in air. Each of these fractions contained a peptide with the same molecular weight as native guanylin (1516 atomic mass units) as determined by mass spectrometric analysis. However, only one of these fractions exhibited potent bioactivity in the T84 cell bioassay consistent with guanylin. This fraction also exhibited a similar HPLC retention time to that of native guanylin. Since guanylin has four cysteine residues, the three fractions of synthetic guanylin probably represented the three possible different disulfide bridge alignments. Bioactive synthetic guanylin stimulated increases in cyclic GMP levels of T84 cells that were time and concentration dependent. Guanylin (10^{-8} M) caused a marked elevation of cyclic GMP after 1 min, which progressively increased through 30 min (Fig. 5*A*). Examination of the concentration–response curve shows that guanylin elicited an increase in cyclic GMP at 10^{-10} M and this response increased through the range of concentrations tested (Fig. 5*B*). To characterize the effect of treatment of reducing agents on the bioactivity of guanylin, we pretreated the peptide for 30 min with 1 mM DTT. The basal level of cyclic GMP for this experiment was 160 ± 50 fmol per well, which increased to 2820 ± 500 fmol per well after a 30-min treatment with guanylin (10^{-8} M). However, following the pretreatment of the peptide with DTT, the effect of the 30-min treatment with the peptide on cyclic GMP was almost completely abolished (250 ± 50 fmol per well). The action of DTT does not appear to be a direct effect of DTT on guanylate cyclase since treatment of the cells with 10 μM DTT (final concentration of DTT that the cells were exposed to in the experiment) failed to affect their responsiveness to STa treatment (data not shown). Finally, we examined in preliminary experiments the ability of guanylin to displace specifically bound ^{125}I-STa from T84 cells. In this experiment, guanylin caused a concentration-dependent displacement of labeled STa from the T84 cells (Fig. 6) with an IC_{50} of 5×10^{-8} M.

DISCUSSION

In the present study, we describe the purification and sequence of a rat intestinal peptide that possesses the properties consistent with an endogenous ligand for the intestinal guanylate cyclase. We have termed this peptide guanylin because of its ability to stimulate intestinal guanylate cyclase. Synthetic guanylin was found to increase cyclic GMP levels in T84 cells in a time- and concentration-dependent manner. Guanylin was also found to displace the specific binding of ^{125}I-STa from T84 cells. Therefore, these data support our proposal that guanylin is an endogenous activator of the

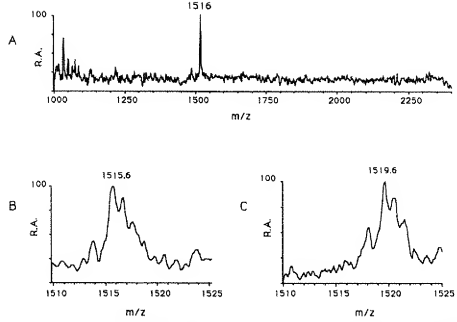

FIG. 3. Electrospray mass spectra of native guanylin. (*A*) Mass spectral analysis of native guanylin in a range of 1000–2400 atomic mass units shows the molecular weight to be 1516 atomic mass units. (*B* and *C*) Comparison of the mass spectra of oxidized (*B*) and reduced (*C*) native guanylin in the range 1510–1525 atomic mass units. R.A., relative abundance.

intestinal guanylate cyclase and suggest that this peptide may influence intestinal fluid and electrolyte transport.

Purification of guanylin was accomplished by capitalizing on the stable nature of this peptide, its acidic isoelectric point, and its characteristic elution on reverse-phase HPLC. Initially, we were concerned that the activity may result from bacterial contamination. To limit this possibility the purification was limited to jejunum, which in normal animals is considered unlikely to contain considerable bacterial contamination. Furthermore, we found in preliminary experiments that every individual rat intestine that we extracted possessed bioactivity. We also found that embryonic intestine, which is considered free of bacteria, exhibits similar activity, strongly suggesting that the intestine indeed possesses a unique ligand. The structure of guanylin further strengthens this proposal. However, definitive proof of the intestinal source of guanylin must await a thorough analysis of the tissue by immunological and molecular methods.

The unique structure of the 15-amino acid peptide guanylin is characterized by an N-terminal proline, a C-terminal cysteine, a glutamic acid, a total of four cysteines, and the absence of basic amino acids. The conditions under which guanylin was isolated may have given rise to a truncated form that possesses the properties required for bioactivity but is derived from a larger precursor. The synthetic peptide required cyclization for expression of bioactivity and this activity was abolished by treatment with the reducing agent DTT. Interestingly, during the purification of oxidized synthetic guanylin, we observed three major chromatographic peaks, each of which contained a peptide of the same molecular weight as native guanylin. Since guanylin has four cysteine residues, the three fractions of synthetic guanylin probably represent the three possible alignments of the disulfide bridges. It is likely that the two inactive fractions represent improperly folded peptide. The alignment of the

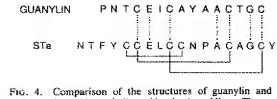

FIG. 4. Comparison of the structures of guanylin and STa. Identical amino acids are indicated by the dotted lines. The reported disulfide alignment for STa (20) is represented by the solid lines.

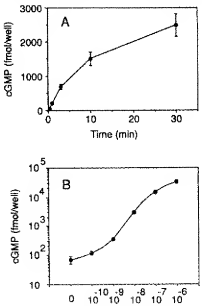

FIG. 5. Time course (*A*) and concentration–response (*B*) effect of synthetic guanylin on cyclic GMP levels in T84 cells. In the time course experiment, T84 cells were treated with 10^{-8} M guanylin for the indicated times. For the concentration–response, the cells were incubated with various concentrations of guanylin for 30 min. Cells for both experiments were treated with 1 mM IBMX. Values represent means ± SE ($n = 4$).

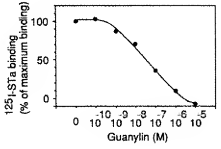

FIG. 6. Displacement of ^{125}I-STa specific binding from T84 cells by guanylin. Cells were incubated for 30 min at 37°C with labeled STa and various concentrations of guanylin. Specific binding (%) was determined by dividing the specifically bound ^{125}I-STa at each guanylin concentration by the specifically bound ^{125}I-STa in the absence of guanylin. Each point represents the mean of triplicates.

disulfide bridges at this time remains undetermined; however, a comparison of the conserved cysteines in guanylin with the known structure of STa (20) suggests that the disulfide bridge alignments may occur between positions 4 and 12 and between 7 and 15 (Fig. 4).

Guanylin appears to act in a manner similar to STs in stimulating cyclic GMP levels and presumably acts through the same extracellular binding region of the intestinal guanylate cyclase. This form of guanylate cyclase has recently been cloned, sequenced, and expressed (5). Intestinal guanylate cyclase was found to possess an extracellular domain that is thought to contain the STa binding region, a transmembrane domain, an intracellular protein kinase-like region, and a cyclase catalytic domain (5). The present study indicates that this protein serves as a receptor for guanylin and mediates the effect of guanylin to increase intracellular cyclic GMP levels. The major site of expression of this receptor appears to be the intestinal epithelial cell, but recent binding studies with ^{125}I-STa indicate that receptors coupled to guanylate cyclase activity exist in other epithelial cells in many different organs of the North American opossum (21, 22). This evidence indicates that this form of guanylate cyclase may be found in other cellular sites than the intestine and may be involved in the regulation of many different cellular functions, particularly epithelial transport. Thus, the actions of guanylin may ultimately extend to other tissues beside the intestine. Indeed, guanylin may possess previously undescribed subtypes of receptors that do not recognize STs.

The immediate physiologic and pathophysiologic implications of the discovery of guanylin primarily relate to the regulation of intestinal fluid and electrolyte transport. A target for guanylin is the intestinal guanylate cyclase and it is likely that this receptor acts to transduce many of the signals for this peptide. Since STa also targets this receptor, the actions of this toxin should serve as a model for the expected actions of guanylin. Guanylin, through its effect on guanylate cyclase and cyclic GMP, may act to decrease sodium and water permeability and to increase chloride secretion. An excess of guanylin would, therefore, be expected to elicit secretory diarrhea in a manner similar to STa. A key to understanding the role of guanylin will be the determination of the specific cell source(s) of guanylin. Thus, the discovery of guanylin should provide a foundation for future studies directed at determining the cellular source of this peptide and its action on epithelial function.

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